MeCP2 Suppresses Nuclear MicroRNA Processing and Dendritic Growth by Regulating the DGCR8/Drosha Complex

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SUMMARY

Loss-of-function mutations of the X-linked gene MECP2 (methyl-CpG binding protein 2) lead to severe neurodevelopmental disorders in humans, such as Rett syndrome (RTT) and autism. MeCP2 is previously known as a transcriptional repressor by binding to methylated DNA and recruiting histone deacetylase complex (HDAC). Here, we report that MeCP2 regulates gene expression posttranscriptionally by suppressing nuclear microRNA processing. We found that MeCP2 binds directly to DiGeorge syndrome critical region 8 (DGCR8), a critical component of the nuclear microRNA-processing machinery, and interferes with the assembly of Drosha and DGCR8 complex. Protein targets of MeCP2-suppressed microRNAs include CREB, LIMK1, and Pumilio2, which play critical roles in neural development. Gain of function of MeCP2 strongly inhibits dendritic and spine growth, which depends on the interaction of MeCP2 and DGCR8. Thus, control of microRNA processing via direct interaction with DGCR8 represents a mechanism for MeCP2 regulation of gene expression and neural development.

INTRODUCTION

Loss-of-function mutations in methyl-CpG binding protein 2 (MECP2) gene are primary causes for Rett syndrome (RTT) (Amir et al., 1999; reviewed in Chahrour and Zoghbi, 2007), whereas the duplications of MECP2-containing loci may lead to autism spectrum disorders in human (Ramocki et al., 2009). Therefore, the dose of MeCP2 protein is critical for the proper development and function of the central nervous system (CNS). MeCP2 was found to primarily bind to methylated CpG islands and acts as a transcriptional repressor by recruiting histone deacetylase complex (HDAC) (Lewis et al., 1992; Nan et al., 1993, 1998; reviewed in Guy et al., 2011). MeCP2 has been shown to play critical roles in regulating gene expression transcriptionally, including brain-derived neurotrophic factor (BDNF) and other genes important for the proper function of the CNS (Chen et al., 2003; Martinowich et al., 2003; Tao et al., 2009; Zhou et al., 2006). Furthermore, MeCP2 was found to regulate synaptic homeostasis by transcriptionally repressing GluA2 expression in an activity-dependent manner (Qiu et al., 2012). Posttranslational modifications of MeCP2 play critical roles in regulating neural development (Cohen et al., 2011). For example, the activity-dependent phosphorylation of Serine 421 (Ser421) of MeCP2 is critical for regulating the recruitment of MeCP2 on DNA and expression of target genes such as BDNF (Cohen et al., 2011; Zhou et al., 2006), and the interaction between MeCP2 and the nuclear receptor corepressor complex (NCoR) is regulated by an activity-dependent phosphorylation of Thr308 of MeCP2 (Ebert et al., 2011; Lyst et al., 2013).

Transcriptome-wide studies revealed that expression of many genes is repressed in the brain of mecp2 null mice, suggesting a positive role for MeCP2 in gene regulation (Chahrour et al., 2008). An alternative possibility is that MeCP2 controls posttranscriptional regulators, e.g., microRNAs (miRNAs), which are known to specifically suppress generation of many proteins that are important for cell proliferation, development, and tumorigenesis (Bartel, 2004; Bushati and Cohen, 2007; Fire et al., 1998). The biogenesis of miRNAs begins with the transcription of the primary miRNAs from the genome, followed by its processing through Drosha/DiGeorge syndrome critical region 8 (DGCR8)-containing nuclear machinery and cytosolic Dicer complex (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Lee et al., 2003). It was reported recently that miRNA expression profile was altered in the brain of mecp2 null mouse due to the transcriptional repression function of MeCP2 (Szlukwach et al., 2010; Urdinguio et al., 2010; Wu et al., 2010). Whether MeCP2 may participate in the miRNA processing directly other than through the transcription process is unknown.

In this study, we found that MeCP2 regulates gene expression posttranscriptionally by regulating nuclear miRNA processing directly. We showed that MeCP2 directly interacts with DGCR8, a critical component of the nuclear miRNA-processing complex, through its C-terminal domain. The phosphorylation of Ser80 of MeCP2 is crucial for binding to DGCR8, which is rapidly dephosphorylated by neuronal calcium signaling. Interestingly, the phosphorylation of Ser80 regulates an intramolecular interaction switch of MeCP2, which leads to an “open” form of the MeCP2 protein and facilitates its binding with DGCR8. We
further provided evidence showing that MeCP2 controls brain-enriched miR-134 processing and thus regulates three of its downstream target genes: cAMP-responsive element-binding protein (CREB), LIM domain kinase 1 (LIMK1), and Pumilio2. These results demonstrate the role for MeCP2 in participating in nuclear miRNA processing directly and suggest a mechanism for which dysregulation of MeCP2 levels leads to neurodevelopmental disorders.

RESULTS

Upregulation of miRNAs in mecp2 Null Mice

To determine whether the miRNA expression profile is altered by MeCP2, we performed Solexa-based RNA sequencing (RNA-seq) to assess global changes in the expression pattern of miRNAs caused by the loss of MeCP2 in mecp2 null (KO) mice (Chen et al., 2001). Normalized with total reads, 314 of all 720 (43.6%) sequenced miRNAs from the hippocampus of mecp2 KO mice were found to be significantly upregulated for over 1.5-fold, as compared to those found in the wild-type (WT) littermates (Figure 1A; Table S1A available online). In contrast, only 63 (8.7%) of them were downregulated. To determine whether overexpression of MeCP2 would inhibit miRNA biogenesis, we applied RNA-seq to mouse cortical neurons with MeCP2 overexpression by lentiviral-based gene delivery. We found that miRNAs are dramatically repressed: 243 of all 373 (65.1%) decreased expression for 1.5-fold with elevated MeCP2 protein level (Table S1B). Among the upregulated miRNAs in mecp2 KO and downregulated miRNAs in MeCP2 overexpression, there are 106 miRNAs overlapped (Tables S1A and S1B). These data indicated that MeCP2 significantly regulates miRNA biogenesis.

Among the upregulated miRNAs in the hippocampus of mecp2 KO mice, we examined the level of 15 primary miRNA transcripts that showed high expression in the CNS and found that 12 of 15 showed no significant changes in the primary transcripts (Figure S1A), suggesting that miRNA upregulation in mecp2 KO mice is most likely caused by the loss of suppressive action of MeCP2 on miRNA processing. To confirm the role of MeCP2 in miRNA processing, we examined the primary, precursor, and mature level of several candidate miRNAs repressed by MeCP2, including miR-134, miR-383, miR-382, and miR-182. We found that primary transcripts of these miRNAs are not altered in neurons expressing either short hairpin RNA (shRNA)
or small interfering RNA (siRNA) against MeCP2, whereas the precursor and mature levels of these miRNAs are significantly increased (Figures 1B–1D and S1B). We further examined the primary, precursor, and mature levels of miR-134 in neurons overexpressing MeCP2 and found that precursor and mature forms of miR-134 were significantly downregulated by MeCP2 overexpression, but not primary miR-134 (Figure S1C), suggesting that MeCP2 regulates the expression of these miRNAs through a transcription-independent mechanism.

Furthermore, we measured the levels of primary and precursor miR-134, one of MeCP2-repressed miRNAs, in the presence of transcriptional blocker actinomycin D when MeCP2 was knocked down by RNAi. We found that the upregulation of miR-134 precursor by MeCP2 RNAi is not affected by actinomycin D treatment, further confirming that MeCP2 represses miR-134 expression in a transcription-independent manner (Figure 1E).

Direct Interaction of MeCP2 with DGCR8
To understand how MeCP2 regulates miRNA processing, we performed glutathione S-transferase (GST) pull-down assays using bacteria-purified MeCP2 to examine the interaction between MeCP2 and components of the nuclear miRNA-processing complex. We found that there was a direct interaction between MeCP2 and DGCR8 (Figure 2A). Previous works showed that the C terminus of MeCP2 may be critical for the interaction between MeCP2 and RNA-binding proteins (Buschdorf and Strätzling, 2004; Young et al., 2005). Frame-shifting and truncated mutations around amino acid 380, which led to a deletion of ~100 aa from the C terminus, are frequently identified mutations

Figure 2. The Direct Interaction between MeCP2 and DGCR8
(A) Direct interaction between MeCP2 and DGCR8 shown by GST pull-down assay. The recombinant GST and GST-DGCR8 purified from bacterial sources were immobilized on glutathione beads, combined with purified His-MeCP2, washed, and analyzed on SDS-PAGE using antibodies indicated. Target bands are indicated by asterisks. WB, western blot.
(B) Interaction between various MeCP2 forms and DGCR8 in HEK293 cells. HA-tagged WT or mutated forms of MeCP2 were coexpressed with myc-tagged DGCR8. Cell lysates were immunoprecipitated with anti-HA antibody and analyzed on SDS-PAGE. MeCP2DN, N terminus (aa 1–91) deleted; MeCP2MBD, methyl-DNA-binding domain (aa 92–161) deleted; MeCP2DN+MBD, both N terminus and MBD (aa 1–161) deleted; MeCP2C, C-terminal segment (aa 380–492) deleted, IP, immunoprecipitation.
(C) Interaction between various DGCR8 forms and MeCP2 in HEK293 cells. Myc-tagged WT, N segment, or C segment of DGCR8 was coexpressed with HA-tagged MeCP2. Cell lysates were immunoprecipitated with anti-myc antibody and analyzed on SDS-PAGE. DGCR8N, (aa 484–773) deleted; DGCR8C, (aa 1–483) deleted.
(D) Direct interaction between MeCP2 and DGCR8 shown by GST pull-down. Procedures similar to those described in (A) were used.
(E) Direct interaction of MeCP2 with both RBD domains of DGCR8, DGCR8N, (aa 511–685) deleted; DGCR8C, (aa 620–750) deleted.
(F) The role of RNA in the interaction of MeCP2 and DGCR8. HA-MeCP2 and myc-DGCR8 were cotransfected into 293T cells. RNase (final concentration of 0.25 μg/ml, 37°C for 30 min) was used to digest RNA prior to immunoprecipitation. Cell lysates were immunoprecipitated with IgG and anti-HA antibody and analyzed on SDS-PAGE. Lower panel shows quantitation of upper panel. Error bars are SEM.
(G) Schematic illustration of interaction domains of MeCP2 and DGCR8.
*p < 0.05 (t test). See also Figures S2 and S3.

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in patients with RTT (Bebbington et al., 2010). The C-terminal deletions accounted for 15% of all genetic mutations identified among the patients with RTT (Bebbington et al., 2010). We found that in lysates of HEK293 cells, the binding of MeCP2 and 380 (a MeCP2 mutant with deletion of residues 380–492) with coexpressed DGCR8 was significantly weaker than that of the WT MeCP2 (Figure 2B). In contrast, binding with DGCR8 was not affected for MeCP2ΔN, MeCP2ΔMBD, and MeCP2ΔN+ΔMBD, which have the N terminus (aa 1–91), methyl-DNA-binding domain (aa 92–161), and both domains deleted, respectively (Nan et al., 1993; Figure 2B). The schematic illustration of domains of MeCP2 and DGCR8 is shown Figures S2A and S2B. Thus, the interaction of MeCP2 with DGCR8 depends on its C terminus, but not the methyl-DNA-binding domain.

To characterize the direct interaction between MeCP2 and DGCR8, we determined the binding affinity between DGCR8 and MeCP2 using Alpha Technology assay. Purified mouse DGCR8C interacted with MeCP2ΔN+ΔMBD with an apparent Kd value of 385 nM. This result further confirms the direct interaction between DGCR8 and MeCP2 (Figures S3A and S3B).

Next, we examined whether MeCP2 is still able to act as a transcriptional repressor. We measured the mRNA level of the BDNF gene, a well-known MeCP2 transcriptional target, in mouse primary cortical neurons (Chen et al., 2003). We found that BDNF mRNA is upregulated when MeCP2 is knocked down by RNAi, consistent with previous findings, and both WT MeCP2 and MeCP2 are able to fully rescue BDNF mRNA to a normal level, indicating that MeCP2 retains its transcriptional repressor activity (Figure S4A; Zhou et al., 2006). We further examined the mRNA level of Acta2, a candidate gene repressed by MeCP2, in mouse primary cortical neurons. We found that Acta2 mRNA level was significantly downregulated by expression of either WT or 380 truncated form of MeCP2, indicating that MeCP2 is able to exhibit transcriptional repression activity as WT MeCP2 (Figure S4B).

To further investigate which domain of DGCR8 is responsible for binding to MeCP2, we generated N- and C-terminal halves of DGCR8, consisting of residues 1–483 (DGCR8ΔN) and 484–773 (DGCR8ΔC), respectively (Figure S2B). Coimmunoprecipitation (coIP) studies showed that the interaction with MeCP2 was reduced for DGCR8ΔN but not for DGCR8ΔC, as compared to full-length DGCR8 (Figure 2C). Furthermore, the GST pull-down experiments with purified proteins showed that MeCP2 directly binds to DGCR8ΔC but not DGCR8ΔN (Figure 2D). Thus, a domain responsible for the interaction with MeCP2 is located in DGCR8ΔC.

Because DGCR8ΔC contains two RNA-binding domains and a C-terminal tail that is required for Drosha binding (Yeom et al., 2006), we prepared mutated forms of DGCR8 with deletions in various domains: DGCR8R2D deleted both RNA-binding domains; and DGCR8R2DΔC, a mutated form, deleted RNA-binding domain 2 and the Drosha-binding C-terminal tail (Figure S2B). We found that DGCR8R2DΔC has no interaction with MeCP2, but deletion of either one of the RNA-binding domains or Drosha-binding domain had no effect (Figures 2E and S4C).

Because RNA-binding domains of DGCR8 are involved in MeCP2 interaction, we also examined whether the presence of RNA is critical for interaction of MeCP2 and DGCR8. We performed an immunoprecipitation experiment with and without ribonuclease (RNase) treatment. Surprisingly, we found that the interaction between MeCP2 and DGCR8 became much stronger when RNAs were digested by RNase, suggesting that MeCP2 may play a negative role in regulating the association of DGCR8 with its RNA substrates (Figure 2F).

These results indicate that both RNA-binding domains of DGCR8 are responsible for directly binding to MeCP2. Interaction domains of MeCP2 and DGCR8 are illustrated in Figure 2G.

**MeCP2 Competes with Drosha for Binding to DGCR8 and RNA**

To explore the mechanism by which MeCP2 regulates nuclear miRNA processing, we tested the possibility that the interaction between MeCP2 and DGCR8 interferes with DGCR8 binding to Drosha, which is the essential RNase III enzyme interacting with the C terminus of DGCR8 in the mRNA-processing complex (Yeom et al., 2006). First, we confirmed by coIP experiments that, in HEK293 cells overexpressing full-length DGCR8, DGCR8ΔN, or DGCR8ΔC, only DGCR8 and DGCR8ΔC bind to endogenous Drosha (Figure 3A). Next, we tested the effect of MeCP2 on the binding of DRCR8 with Drosha. We coexpressed DGCR8 with either full-length MeCP2 or MeCP2 in HEK293 cells, and coIP results showed that the binding of DGCR8 with Drosha was significantly weaker in the presence of the full-length MeCP2, but not MeCP2 (Figure 3B). We further confirmed this finding by examining the interaction between DGCR8 and Drosha in the presence of MeCP2 in a dose-dependent manner. We coexpressed DGCR8 with an increasing amount of MeCP2 and found that the interaction between DGCR8 and Drosha appeared to decrease when MeCP2 level increased (Figure 3C). These results indicate that MeCP2 interaction with DGCR8 suppresses its binding with Drosha. The RNA-binding domains of DGCR8 may be involved in MeCP2 binding, and the essential Drosha-binding C-terminal tail is close to the RNA-binding domains (Yeom et al., 2006). Therefore, the suppressive action of MeCP2 may be caused by a direct competition with Drosha for DGCR8 binding and/or a steric hindrance of MeCP2 on Drosha binding with the DGCR8 C-terminal tail. Together, these results strongly suggest that MeCP2 plays a role in interfering with DGCR8–RNA association, as well as the DGCR8–Drosha interaction, and thereby suppresses DGCR8/Drosha-mediated miRNA processing.

**Phosphorylation of MeCP2 at Ser80 Facilitates MeCP2 Binding with DGCR8**

Phosphorylation of MeCP2 at Ser80 under resting status is rapidly dephosphorylated upon neuronal activity (Tao et al., 2009; Zhou et al., 2006). Through coIP experiments in HEK293 cells coexpressing DGCR8 and various forms of MeCP2, we found that DGCR8 binding with MeCP2 was significantly lowered when Ser80 of MeCP2 was mutated to phosphorylation-deficient alanine (MeCP2) but elevated when it was mutated to phosphorylation-mimicking aspartate (MeCP2, Figure 3D).

We reproduced a previous finding that MeCP2 is quickly dephosphorylated at the Ser80 site after depolarization (Figure 3E; Tao et al., 2009). To investigate whether the interaction between MeCP2 and DGCR8 may be regulated by neuronal activity, we thus performed a coIP assay with endogenous proteins in primary culture neurons. We found that the interaction of MeCP2
with DGCR8 from lysates of cultured mouse cortical neurons decreased significantly after depolarization (Figure 3F).

Next, we investigated whether the interaction of DGCR8 with Drosha would be affected by MeCP2 in neurons. After knocking down MeCP2 by RNAi, we found that the interaction between DGCR8 and Drosha dramatically increased in mouse cortical culture neurons (Figure 3G). Consistently, interaction between DGCR8 and Drosha appeared to significantly decrease in a mecp2 transgenic (TG) mice model where mecp2 was strongly overexpressed genetically, compared to WT littermates (Figure 3H; Collins et al., 2004). Thus, this evidence strongly suggests that MeCP2 interferes with the assembly of the

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A

\[ \text{MeCP2 WT} \quad \text{MeCP2 S80A} \quad \text{MeCP2 S80D} \]

\[ p < 0.001 \]

B

\[ \text{Un} \quad \text{KCl} \]

\[ p = 0.0233 \]

C

\begin{array}{ccc}
\text{Input} & \text{GST pull-down} \\
\text{GST-MeCP2-N} & \text{--} & \text{--} & \text{--} & \text{--} & \text{+} \\
\text{GST} & \text{--} & \text{+} & \text{--} & \text{+} & \text{--} \\
\text{His-MeCP2-C} & \text{+} & \text{+} & \text{+} & \text{+} & \text{+} \\
\end{array}

D

\begin{array}{c}
\text{myc-MeCP2-C} \\
\text{anti-HA IP} \\
\text{IgG} \\
\text{WB} \\
\text{myc} \\
\text{Input} \\
\text{Relative level} \\
\text{normalized to HA input} \\
\text{HA-MeCP2N-S80A} \\
\text{HA-MeCP2N-S80D} \\
\end{array}

E

\[ 407\text{nm} \quad 475\text{nm} \]

Calcium-dependent dephosphorylation of S80

\[ 407\text{nm} \quad 527\text{nm} \]

FRET

\( \text{legend on next page} \)
Intramolecular Interaction of MeCP2 Protein Regulated by the Phosphorylation Status of Ser80

However, as we showed, the N-terminal region of MeCP2 is not required for binding with DGCR8. To address how phosphorylation of Ser80 contributes to the interaction between MeCP2 and DGCR8, we used fluorescence resonance energy transfer (FRET)-based analysis to examine whether phosphorylation of Ser80 would alter the conformation of MeCP2, instead of directly interacting with DGCR8 (Hillebrand et al., 2007). We tagged two distinct fluorescent proteins, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), onto the N and C terminus of MeCP2, respectively. The CFP-MeCP2-YFP probe expressed strictly in the nucleus of both 293T cells and mouse primary cortical neurons, as shown by our immunostaining results (Figure S4D). We found that the FRET signals increased when the Ser80 was mutated to phosphorylation-deficient alanine, compared to WT and the MeCP2S80D mutant, because Ser80 of WT MeCP2 is phosphorylated in basal condition in 293T cells (Figures 4B and S4E). These data suggest that there may be intramolecular interactions between the N- and C-terminal halves of unphosphorylated MeCP2, which restrict the accessibility of MeCP2 by DGCR8, and that phosphorylation of Ser80 of MeCP2 opens up the conformation and facilitates its binding to DGCR8, as schematically shown in Figure 4E. We further performed the FRET experiments with the CFP-MeCP2-YFP probe in neurons with and without depolarization stimulus. We found that FRET efficiency of the CFP-MeCP2-YFP probe was increased upon potassium chloride (KCl) stimulus (Figure 4B), supporting that there is a conformational change in MeCP2 protein upon neuronal activity.

To examine this potential intramolecular interaction further, we performed a GST pull-down assay using bacterially purified GST-tagged N (residues 1–305) and His-tagged C (residues 306–492) terminal halves of MeCP2. We found that there was a direct interaction between GST-MeCP2-N and His-MeCP2-C (Figure 4C). Furthermore, we examined the role of Ser80 phosphorylation in the interaction between MeCP2-N and MeCP2-C using a coIP assay in 293T cells. We found that phosphorylation-deficient MeCP2-N-S80A binds to MeCP2-C in a much stronger fashion, compared to phosphorylation-mimicking MeCP2-N-S80D (Figure 4D). These data indicate that, in the Ser80 unphosphorylated state, MeCP2 forms an intramolecular interaction between its N- and C-terminal halves, which makes the C-terminal half inaccessible for binding to DGCR8.

Taken together, we propose that phosphorylated MeCP2 binds to DGCR8 under resting status in neurons. When neuronal calcium signaling is activated, dephosphorylation of MeCP2 Ser80 leads to its release from DGCR8 and allows activity-dependent miRNA processing to take place (Figure 4E).

MeCP2 Regulates CREB, LIMK1, and Pumilio2 Protein Levels via Suppressing miR-134

To further determine the role of phosphorylation of MeCP2 Ser80 in miRNA processing, we focused on the miR-134, a critical miRNA for neural development (Schratt et al., 2006; Gao et al., 2010). We measured primary, precursor, and mature forms of miR-134 under different manipulations of MeCP2. First, we found that the precursor and mature forms of miR-134, but not primary transcript, were significantly decreased in neurons expressing WT MeCP2, whereas overexpression of the truncated form MeCP2S80D has no effects on miR-134 levels (Figure 5A).

Consistently, we found that overexpression of phosphorylation-mimicking MeCP2S80D significantly decreased precursor and mature miR-134, but MeCP2S80A has no effect (Figures 5B and 5C). Thus, both our loss- and gain-of-function data indicate that phosphorylation of MeCP2 Ser80 is required for repressing miR-134 processing.

To investigate the functional consequences of MeCP2 regulation of miR-134 processing, we further examined whether the level of targeted proteins of MeCP2-suppressed miR-134 is altered in the absence of MeCP2. It was reported that miR-134 targets three critical regulators for neural development and plasticity: CREB, LIMK1, and Pumilio2 (Fiore et al., 2009; Gao et al., 2010; Schratt et al., 2006). We found that overexpression of MeCP2 in mouse primary cortical neurons indeed leads to an increase of CREB level (Figure 5D). Interestingly, when we coexpressed DGCR8 along with MeCP2 in cortical neurons, we found that the introduction of DGCR8 could neutralize the effect of MeCP2 overexpression and rescue the CREB to the normal level (Figure 5D), suggesting that MeCP2-induced CREB upregulation depends on interaction of MeCP2 and DGCR8. Consistently, we found that the level of mature miR-134 was indeed repressed by MeCP2 overexpression and rescued by coexpression of DGCR8 (Figure 5E). Furthermore, we found that the expression of the MeCP2S80D-truncated form was not able to increase CREB level

Figure 4. Intramolecular Interactions in MeCP2 Protein Regulated by Phosphorylation of Ser80

(A) FRET efficiency is increased in S80A mutant compared with the WT MeCP2 and MeCP2S80D. CFP and YFP are tagged onto N and C termini of WT MeCP2, MeCP2S80A, and MeCP2S80D. Fluorescence dot signals from ten cells of each condition were collected and measured. p < 0.001 (Kolmogorov-Smirnov test).

(B) FRET efficiency is increased in MeCP2 FRET probe upon depolarization in neurons. CFP and YFP are tagged onto N and C termini of WT MeCP2 and are transfected into mouse primary cortical neurons. Under depolarization conditions, neurons were stimulated with 50 mM KCl for 3 hr. Fluorescence signals from ten cells of each condition were collected and measured. p = 0.023 (Kolmogorov-Smirnov test).

(C) Direct interaction between GST-MeCP2-N (aa 1–305) and His-MeCP2-C (aa 306–492) shown by GST pull-down assay. The recombinant GST and GST-MeCP2-N purified from bacterial sources were immobilized on glutathione beads, combined with purified His-MeCP2-C, washed, and analyzed on SDS-PAGE. Lower panel shows quantification of (D). Error bars are SEM. *p < 0.05 (t test).

(E) In the unphosphorylated state (or the S80A mutant), the accessibility of the MeCP2 C-terminal domain (CTD) is restricted by a potential intramolecular interaction, between the MeCP2 CTD and the MeCP2 N-terminal region including S80. Phosphorylation of S80 increases the CTD accessibility, likely by disrupting this intramolecular inhibition of MeCP2 and forming an open conformation. See also Figure S4.
in cortical neurons as WT MeCP2 (Figure 5F). These data indicate that the interaction with DGCR8 is critical for MeCP2 to posttranscriptionally regulate CREB level.

We found that the CREB and LIMK1 protein levels were significantly elevated when the WT MeCP2, but not MeCP2<sub>S80A</sub>, was overexpressed in cultured neurons, consistent with our finding...
that MeCP2S80A showed much weaker interaction with DGCR8 than WT MeCP2 (Figure 5G). Next, we found that the expression of WT MeCP2 with same-sense mutations of shRNA-targeting sites in these neurons rescued the reduction of CREB and LIMK1 caused by MeCP2 RNAi, whereas the expression of MeCP2S80A also containing same-sense mutations had no effect on CREB and LIMK1 protein levels (Figure 5H). These data indicate that the phosphorylation of the MeCP2 S80 site is essential for upregulation of CREB level by MeCP2 overexpression, further suggesting that interfering with miRNA processing by MeCP2 could lead to multiple physiological consequences by affecting target gene expression.

Importantly, we found that the protein levels of CREB and LIMK1 were also decreased by downregulating MeCP2 with expression of specific shRNA in cultured cortical neurons, which was largely blocked by introducing the miR-134 inhibitor that was complementary to the mature miR-134 (Figure 5I), strongly suggesting that miR-134 is required for MeCP2 regulating CREB and LIMK1 protein levels. Notably, the levels of primary miR-134 transcripts, CREB, and LIMK1 remain constant under these manipulations, indicating that the regulation by MeCP2 on these components occurs at the posttranscriptional level (Figures S5A–S5F).

We also examined the protein level of Pumilio2 under different manipulations of MeCP2. Consistently, we found that Pumilio2 protein level decreases in MeCP2 RNAi and increases in MeCP2 overexpression samples (Figure S5G). The mRNA levels of Pumilio2 are not altered in either MeCP2 RNAi or overexpression conditions, compared to control (Figure S5H).

To determine whether miR-134 and its target proteins would be regulated in vivo, we examined levels of mature miR-134, CREB, LIMK1, and Pumilio2 proteins in hippocampal lysates of MeCP2 KO and TG mice, using WT littermates as control (Chen et al., 2001; Collins et al., 2004). First, we found that the mature miR-134 level significantly increased in brain lysates of mecp2 KO, whereas it decreased in those of mecp2 TG, compared to WT mice (Figure 5J). Next, we found that the level of CREB and LIMK1 dramatically decreased in the hippocampus of mecp2 KO mice as compared to WT (left panel of Figure 5K). On the other hand, we found that CREB and LIMK1 protein levels notably increased in mecp2 TG mice (right panel of Figure 5K). This is consistent with the previous finding that the protein level of CREB was increased in the hypothalamus of mecp2 TG mice compared to WT mice (Chahrour et al., 2008). Importantly, mRNA levels of primary miR-134, CREB, and LIMK1 were not affected in either mecp2 KO or TG mice (Figures S6A–S6F). We also found that the protein level of Pumilio2 decreased in the hippocampus of mecp2 KO and increased in the hippocampus of mecp2 TG mouse (Figure 5L). Taken together, these data indicate that MeCP2 posttranscriptionally controls levels of miR-134, CREB, LIMK, and Pumilio2 protein in vivo.

### Repression of Dendritic Growth by MeCP2 via Controlling miRNA Processing

Next, we would like to address how the interaction of MeCP2 with DGCR8 contributes to neural development. The duplications of MECP2-containing loci lead to severe autism spectrum disorders in human, suggesting that gain-of-function mutations of MECP2 may cause destructive consequences to the development of the nervous system (Ramocki et al., 2009). Consistently, it was reported that dendritic growth of hippocampal neurons is inhibited when MeCP2 is elevated and TG mouse with mecp2 overexpression appears to have phenotypes mimicking the MECP2 duplication disorders (Zhou et al., 2006). Furthermore, CREB, LIMK1, and Pumilio2, these target genes of MeCP2 shown above, were reported to play critical roles in regulating dendritic growth (Redmond et al., 2002; Schratt et al., 2006; Vessey et al., 2010).

We would like to address whether this inhibition of dendritic growth by MeCP2 is due to the repression of miR-134. We examined the dendritic growth of the mouse primary cortical culture neurons and found that expression of WT MeCP2, not MeCP2S80A and MeCP2S80D, leads to strong inhibition of dendritic growth (Figures 6A and 6B). We coexpressed miR-134 and its antisense control along with WT MeCP2 in cultured neurons. Strikingly, we found that expression of miR-134 was able to fully rescue the dendritic growth defects caused by WT MeCP2 overexpression, but miR-134 antisense control had no effect (Figures 6A and 6B). We expressed miR-134 and the MeCP2 RNAi construct alone in the cortical neurons and found that there were no significant changes in dendritic growth (Figures S7A–S7D). These data indicate that MeCP2 represses neuronal dendritic growth by suppressing miR-134.

Next, we asked whether the calcium-dependent dephosphorylation of MeCP2 Ser80 may contribute to the well-characterized activity-dependent dendritic growth process in neurons (Redmond et al., 2002). We transfected WT MeCP2 and MeCP2S80D mutation into mouse primary cortical neurons and depolarized the neuron with KCl. We found that the activity-dependent dendritic growth was completely abolished only when the S80D mutated form of MeCP2 was introduced, indicating that calcium-dependent dephosphorylation of MeCP2 Ser80 is required for activity-dependent dendritic outgrowth (Figures 6C and 6D).

Finally, we examined the role of MeCP2 in dendritic growth and spine development in hippocampal slice culture preparations. We used gene gun-based DNA delivery on rat postnatal day 7 (P7) hippocampal slice cultures and found that introduction of WT MeCP2 significantly decreased dendritic growth, as measured by total dendritic length (Figures 7A and 7B). However, overexpression of two mutant forms of MeCP2, MeCP2S80A and MeCP2S80D, had no such effect (Figures 7A and 7B). Note that MeCP2S80A and MeCP2S80D are not able to bind to DGCR8 and repress miRNA processing. Furthermore, we measured spine density in the above preparations and found that overexpression of WT MeCP2, but not MeCP2S80A and MeCP2S80D, strongly repressed spine density in hippocampal slice culture, further suggesting that the role of MeCP2 on spine development also requires the interaction of MeCP2 and DGCR8 (Figures 7C and 7D; Han et al., 2013; Schratt et al., 2006). Taken together, these results indicate that the gain of function of MeCP2 leads to inhibition of dendritic and spine growth, depending on the repression of DGCR8/Drosha-dependent miR-134 processing by MeCP2.

### DISCUSSION

In this study, we have identified a mechanism by which MeCP2 regulates gene expression by suppressing nuclear miRNA
processing through direct interaction with DGCR8, a key component of the processing complex. This mechanism could contribute significantly to the MeCP2’s regulation of proteins that play important neuronal functions, e.g., CREB, LIMK1, and Pumilio2.

Here, we showed that as a nuclear protein, MeCP2 could not only repress gene transcription by binding to methylated DNA and recruiting transcriptional repressors (Nan et al., 1998) but also suppress miRNA processing by binding to RNA-binding domains of DGCR8. Whether MeCP2 may interact with other RNA-binding proteins and participate in RNA biogenesis and metabolism needs to be further investigated. The previous study that MeCP2 interacts with the RNA-splicing complex suggests

Figure 6. MeCP2 Regulates Dendritic Growth by Inhibiting miR-134, and Dephosphorylation of MeCP2 S80 Is Required for Activity-Dependent Dendritic Growth

(A) Example picture of mouse primary cortical neurons transfected at 3 DIV with GFP alone and GFP with constructs indicated. Neurons were fixed and immunostained with GFP antibody at 6 DIV for measurement of dendritic length.

(B) Measurements of total dendritic length of each condition. *p < 0.01 (t test).

(C) Example picture of mouse primary cortical neurons with each construct transfected. Constructs expressing MeCP2 WT and mutations were transfected into cortical neuron 2 DIV and stimulated by KCl 48 hr after transfection. KCl with a final concentration of 25 mM was given to mouse primary cortical neurons for 36 hr before neurons were fixed and performed following experiments. Immunostaining with GFP antibody was used to examine the dendritic growth.

(D) Measurements of total dendritic length of each condition. *p < 0.01 (t test). A total 12–15 neurons from each condition were randomly selected and measured. See also Figure S7.
that MeCP2 may be widely involved in RNA metabolism processes (Young et al., 2005).

How miRNA biogenesis in neurons is regulated by neuronal activity is an important question. A previous report showed that light-induced neuronal activity is critical for fast turnover of mature miRNAs in retina cells (Krol et al., 2010). Our study suggests that miRNA biogenesis may be positively regulated by neural activity, as controlled by calcium-dependent dephosphorylation of MeCP2 and derepressing of miRNA processing. Thus, this work may represent regulation over different levels or in different tissues. We suggest that MeCP2-regulated nuclear miRNA processing plays a critical role for miRNA biogenesis in cortical and hippocampal neurons.

It is important that nuclear miRNA processing is critical for proper synaptic function as shown by Ullian and colleagues that deletion of DGCR8 leads to defects of inhibitory synaptic transmission (Hsu et al., 2012). Whether MeCP2 may be involved in inhibitory synaptic transmission via controlling nuclear miRNA processing would be an interesting question to address.

An important issue regarding the pathophysiology of MeCP2-related disorders is that both loss and gain of functions of MeCP2 lead to severe pathological symptoms. Loss-of-function mutations of MECP2 lead to RTT, whereas duplication of MECP2-containing locus leads to autism spectrum disorders. It is difficult to provide a satisfactory explanation for why a higher level of this protein leads to diseases, if solely considering the transcriptional regulation point of view. Our study showed that MeCP2 could regulate expression levels of target genes by fine-tuning the level of miRNAs, as shown for CREB, LIMK1, and Pumilio2 protein levels. Thus, this mechanism provides an alternative explanation for the dose effect of MeCP2 in neural development. As we showed in the Results, increasing the amount of MeCP2 protein leads to repression of mature miR-134, which then causes various dysregulation of protein targets as well as cellular consequences.
Nuclear miRNA processing is a highly regulated process, as exemplified by the effects of p53 and SMAD proteins that are involved in tumorigenesis (Davis et al., 2008; Suzuki et al., 2009). Our finding of a posttranscriptional function of MeCP2 in regulating miRNA maturation suggests that intervening dysregulated miRNA processing represents a potential therapeutic approach in the treatment of RTT.

**Experimental Procedures**

### Plasmids

The rat MeCP2-e2 gene was a gift of Dr. Adrian Bird. Other constructs of MeCP2 mutations and truncations were all generated based on this construct. In brief, MeCP2Δ250, MeCP2Δ250ΔC, MeCP2Δ250ΔDA, and MeCP2Δ340 were generated using KOD-Plus (ToyoBio) according to manufacturer’s instructions. The mouse MeCP2 shRNA was directed against the sequence as follows: 5'-AAGTCTCA AAACGCGAGATGACCT-3'. MeCP2 siRNAs (Thermo Scientific, against mouse MeCP2, on-target plus SMART pool L-044034-09-12, 5'-CCGAAUUGCCUGCUUUA-3', 5'-CCGAUUGCUUGCUUUA-3', and 5'-CCGAUUGGCUUGCAGUAAUGCUUUA-3'), along with scrambled siRNA as control, mir-134 sequence was amplified with primers as the following: forward, 5'-cagggattaacccactgtgagcaggacgcg-3', and reverse, 5'-cagggattaacccactgtgagcaggacgcg-3'. The miR-134 sequence or reverse complement sequence was inserted into FUGW to get miR-134 plasmid/miR-134rev plasmid. mmu-mir-134 inhibitor was purchased from GenePharma.com. Inhibitors are 2'-methylated-modified RNA oligo completely complementary to mmu-mir-134 and purified by high-performance liquid chromatography prior to being used. mir-134 inhibitors were introduced into mouse neurons by Amaxa Nucleofector at the time of seeding. The mouse DGCR8 cDNA was amplified from total cDNA of mouse cortical neurons. DGCR8 truncations were generated as described previously by Yeom et al. (2006).

### Antibodies

Antibodies used in this study were as follows: MeCP2 (#3456, Cell Signaling Technology), CREB (#9197; Cell Signaling Technology), DGCR8 (sc-48473; Santa Cruz Biotechnology); Myc (9291; Cell Signaling Technology; MP4591, MP4601, ECM Biosciences); Drosha (#3364; Cell Signaling Technology); MeCP2 (antibody #2622; Cell Signaling Technology); CREB (#2622; Cell Signaling Technology); GAPDH (ab8245; Abcam). Antibodies used in this study were as follows: MeCP2 (#3456, Cell Signaling Technology; MP4591, MP4601, ECM Biosciences); Drosha (#3364; Cell Signaling Technology; GDCRB (sc-48473; Santa Cruz Biotechnology); Myc epitope tag (#M20002; Abmart); HA epitope tag (#M20013; Abmart); GST (#2622; Cell Signaling Technology); CREB (#9197; Cell Signaling Technology); LIMK1 (#8342; Cell Signaling Technology); and GAPDH (ab8245; Abcam).

### Cell Culture

Embryonic day 15 (E15)–E16 mouse cortical cells were cultured and transfected with Amaxa Nucleofector in 6-well plates. Cells were collected at 5–7 days in vitro (DIV) for further analysis. Lentivirus used in this study was made with 10^5–10^6 viral genome/ml by the Obio Technology (Shanghai) and the Neonobiotech.

### Genetic-Modified Mice

mecp2 Tg mice (008679) and nestin-cre mice (003771) were purchased from Mutant Mouse Regional Resource Center at University of California Davis. The use and care of animals complied with the guideline of the Animal Advisory Committee at the Shanghai Institutes for Biological Science, CAS.

### Quantitative Real-Time PCR Assays

For quantitative real-time PCR assays, total RNA was extracted from mouse cortical neurons using mirVana miRNA Isolation Kit (Ambion). For the measurement of primary miRNA transcripts, the large-sized RNA fractions (>200 bp) were used for reverse transcription. cDNA was synthesized by poly-dT primers and cDNA was amplified from total cDNA of mouse cortical neurons. DGCR8 truncated cDNAs were generated as described previously by Yeom et al. (2006). overnight express TB medium from Novagen (catalog #71491-4) was used to generate recombinant protein from the Neuronbiotech.

### GST Pull-Down Assay

GST pull-down assay was performed as described before (Qiu and Ghosh, 2008). Overnight express TB medium from Novagen (catalog #71491-4) was used to generate recombinant protein from *E. coli*.
was then incubated overnight at 4°C with primary antibodies, washed three times in TBST, and the signals were revealed by horseradish peroxidase reaction using the SuperSignal Chemiluminescent Substrate (Pierce).

RNA-Seq Deep Sequencing
Hippocampus RNA samples were prepared from P30 WT and KO mice and were sequenced and analyzed by Solexa of BGI Shenzhen. RNA-seq sample preparation and data analysis were performed as described by Kwak et al. (2009). RNA from cultured mouse cortical neurons with MeCP2 manipulations and depolarization stimulus were collected 5–7 DIV and sequenced by Solexa GAII of Shanghai Bio-Chip. Standard Solexa TruSeq-Small RNA sequence protocol was used. The technical method is described in the TruSeq Small RNA Sample preparation guide on the illumina website. CLC Genomics Workbench V.5.5 was used to perform miRNA data analysis. RNA-seq data analysis was performed by Novel Bioinformatics.

FRET Assay
FRET assays were performed as described by Hillebrand et al. (2007) with modifications. 293 cells were transfected with CFP-MeCP2, MeCP2-YFP, CFP-MeCP2-YFP, and CFP-MeCP2AA-YFP and fixed after 48 hr. Primary cultured cortical neurons were transfected in the same way and stimulated with KCl for 3 hr before fixation. All quantified FRET data were collected using 514 nm laser lines, and emission was collected at 540 nm. For each condition, and the total length of all protrusions was analyzed using Fiji software.

Supplemental Information
Supplemental Information includes seven figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.01.032.

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